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ABSTRACT Evidence links dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) to crucial military health issues, including operational stress, resilience, and traumatic brain injury. This study evaluated the anabolic, neuroprotective, and neuroexcitatory properties of DHEA(S) in healthy military men. A salivary sample was obtained from 42 men and assayed for DHEA(S), testosterone, nerve growth factor (NGF; which supports nerve cell proliferation), and salivary alpha amylase (sAA; a proxy of sympathetic nervous system function). Separate regression analyses were conducted with DHEA and DHEAS as independent variables, and testosterone, NGF, and sAA as dependent variables, respectively. The models explained 23.4% of variance in testosterone ($p < 0.01$), 17.2% of variance in NGF ($p < 0.01$), and 7.4% of variance in sAA ($p = 0.09$). Standardized beta coefficients revealed that DHEA independently influenced testosterone ($\beta = 0.40$, $p < 0.01$), whereas DHEAS independently influenced NGF ($\beta = 0.48$, $p < 0.01$) and sAA ($\beta = 0.36$, $p < 0.05$). DHEA demonstrated anabolic properties, whereas DHEAS demonstrated neuroprotective and neuroexcitatory properties in military men. This area of study has broad implications for stress inoculation, traumatic brain injury rehabilitation, and regenerative medicine in military personnel.

INTRODUCTION

Dehydroepiandrosterone (DHEA) and its sulfate ester dehydroepiandrosterone sulfate (DHEAS) (collectively referred to as DHEA[S]) are cosecreted with cortisol from the adrenal cortex, whereas DHEA is further produced by neurons and glia within the brain.¹ Their precise mechanisms of action are not fully understood. Preclinical evidence suggests that these steroids precurse the sex steroids testosterone and estrogen^{2,3}; confer neuroprotection as a result of antiglucocorticoid/antitoxin action^{4,5} and interaction with neurotrophins⁶; and also stimulate the sympathetic nervous system via both gamma-aminobutyric acid (GABA) inhibition⁷ and glutamate activation.⁸ Little available data, however, quantify these properties in humans. A better understanding of these relationships may have broad implications for stress inoculation, traumatic brain injury (TBI) rehabilitation, and regenerative medicine in military personnel. This cross-sectional study examined anabolic, neuroprotective, and neuroexcitatory properties of DHEA(S) in healthy, free-living military men.

DHEA(S) and Steroidogenesis

Perhaps the best known function of DHEA(S) is its role as a weak precursor to the sex steroids testosterone and estrogen as evidenced in the well-characterized human steroidogenesis

pathway.^{2,3} Specifically, DHEA is converted via one major pathway to androstenedione, catalyzed by 3β -hydroxysteroid dehydrogenase [HSD]. Androstenedione is then converted to testosterone, catalyzed by 17β -HSD. The strength of relationship between DHEA(S) and testosterone concentrations in humans as evidenced by cerebrospinal fluid, serum, or salivary sampling, is surprisingly understudied. Likewise, the relative contributions of DHEA and DHEAS to testosterone production are not known. There is a need for translational research exploring these relationships in humans.

DHEA(S) and Neuroprotection

Other evident effects of DHEA(S) include neuroprotection,^{4,9} neurite growth,¹⁰ and neurogenesis.¹¹ Regarding neuroprotection, DHEA(S) is believed to exert prosurvival effects by modulating GABA,^{4,12} glutamate,⁸ *N*-methyl-D-aspartate (which mimics action of glutamate), and/or sigma-1 receptors (implicated in brain plasticity)¹³; or possibly after it is converted to the sex steroids testosterone and estrogen.⁶ Recent preclinical evidence also suggests that DHEA operates in conjunction with and/or via direct action upon members of the neurotrophin family, such as neurotrophin-3, brain-derived neurotrophic factor, and nerve growth factor (NGF).^{6,14} At least two mechanisms describing the DHEA-NGF interface have emerged from the animal literature. In the first, DHEA binds with transmembrane NGF receptors tyrosine kinase-A (TrkA) and p75 neurotrophin receptor (p75^{NTR}) on target cells, the balance of which ignites a sequence of events modulating expression and function of proteins governing apoptosis (i.e., cell death) (Fig. 1). Evidence that DHEA binds directly to these receptors and prevents neuronal apoptosis has been shown in sensory neurons and sympathetic neurons in mice and rats, respectively.⁶ As Lazaridis et al stated (p. 10), the

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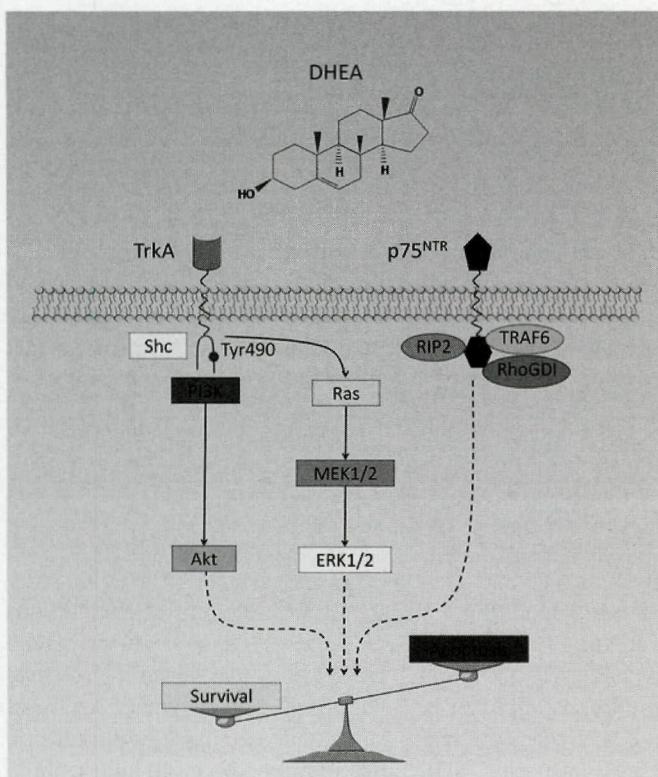


FIGURE 1. Hypothesized effect of DHEA(S) on TrkA and p75^{NTR} of sensory and sympathetic neurons.⁶

“decision between survival and death” among DHEA-responsive cells is determined by the balance of DHEA’s interaction with these two receptors. In the second mechanism, DHEA is believed to upregulate NGF messenger RNA (mRNA) expression in target cells. Evidence for this fascinating process has been shown in primary rat hippocampal cultures, whereby DHEA upregulated NGF mRNA after 3 hours of exposure while opposite effects were noted for the stress hormone cortisol.¹⁴ This latter observation is consistent with the frequently reported but imperfectly understood antagonistic relationship between DHEA(S) and cortisol.¹⁵ Studies addressing relationships between DHEA(S) and NGF in humans are sparse. In one exception, Schulte-Herbrüggen et al¹⁶ showed no relationships between serum DHEAS and NGF concentrations in a study of 40 pregnant women. To this author’s knowledge, the DHEA(S)-NGF association has not been quantified in healthy human male populations.

DHEA(S) and Neuroactivation

DHEA(S) is also believed to have neuroexcitatory properties, in part because of negative modulation of GABA, the chief inhibitory neurotransmitter in the mammalian nervous system that acts principally on norepinephrine,¹⁷ epinephrine, and neuroendocrine systems.¹⁸ Specifically, animal models suggest that DHEA(S) interacts with the GABA_A receptor complex on target neurons throughout the brain and central nervous system, inhibiting GABA-mediated neurotransmis-

sion, thus initiating a net “excitatory” effect.⁷ Some of these studies further suggest that DHEAS modulates this receptor complex with greater potency than DHEA.^{19,20} Direct relationships between DHEA(S) and sympathetic nervous activity in humans are not well-characterized.

DHEA(S) Applications in Military Populations: Operational/Traumatic Stress and TBI

Recent studies demonstrate stress inoculation effects of endogenous DHEA(S) during military stress,^{21,22} specifically evidencing buffered acute stress symptoms and/or performance maintenance. Morgan et al,²¹ for example, showed that plasma DHEA(S) correlated to improved performance and fewer dissociative symptoms in a stressful underwater navigation exam in military members enrolled in a combat diver qualification course. Regarding exogenous (administered) DHEA, Taylor et al²² recently found that a brief, low-dose DHEA regimen yields dramatic increases in salivary DHEA(S) concentrations and enhances anabolic balance during military stress, but no effects were observed with respect to subjective distress.

Animal models also suggest exciting potential use for DHEA supplementation in the clinical treatment of TBI.^{23,24} Hoffman et al²³ demonstrated in a rat model that delayed administration of DHEAS improves behavioral performance recovery from induced TBI on both sensory and cognitive tasks. Likewise, Malik et al²⁴ showed convincing effects of a DHEA analogue (fluasterone) in improving functional recovery (e.g., balance, neurological reflexes) from induced TBI.

In sum, preclinical evidence suggests anabolic, neuroprotective, and neuroexcitatory effects of DHEA(S), but few studies have quantified these relationships in humans. The current study was designed to evaluate the anabolic, neuroprotective, and neuroexcitatory properties of DHEA(S) in healthy military men. Accordingly, it was hypothesized that DHEA(S) would positively associate with the anabolic hormone testosterone, the neurotrophin NGF, and the sympathetic analogue salivary alpha amylase (SAA).

METHODS

Subjects

Subjects included 42 healthy, male, active duty Navy and Marine Corps personnel (mean ± SD age 26.4 ± 4.6 years) who had reported to Naval Air Station North Island to begin Survival, Evasion, Resistance, and Escape (SERE) training. This same sample was also studied in a prospective evaluation of DHEA supplementation during survival training, and those findings are reported elsewhere.²² Subjects who were deemed medically fit to undergo SERE training and were enrolled in the SERE course were thus considered eligible for the current study, with two exceptions: women were excluded because of health concerns associated with DHEA supplementation, as were individuals who endorsed taking any anabolic or ergogenic supplement within the past 3 months or who were

currently taking any over-the-counter medications. Those who expressed an interest in participating attended an in-person meeting to review the details of the study and provide written informed consent. This protocol was approved by the Naval Aerospace Medical Research Laboratory Institutional Review Board.

Salivary Sampling

For this study, a single salivary sample was obtained via the passive drool method²⁵ between 1145 and 1247 under baseline, free-living conditions on the first day of academic (classroom) instruction for military survival training. Each subject was asked to rinse his mouth with water approximately 10 minutes before sample collection and to avoid the following: brushing teeth before collection, using salivary stimulants (e.g., gum, lemon drops), and consuming acidic or high-sugar foods within 20 minutes before collection. After data collection, all samples were immediately placed on dry ice and transferred to Salimetrics, LLC (State College, Pennsylvania) for storage and data processing. Samples were assayed for DHEA, DHEAS, testosterone, NGF, sAA, and cortisol.

DHEA and DHEAS

All samples were assayed for salivary DHEA in duplicate using a highly sensitive enzyme immunoassay. The test uses 50 µL of saliva per determination, has a lower limit of sensitivity of 5 pg/mL, standard curve range from 10.2 pg/mL to 1,000 pg/mL, an average intra-assay coefficient of variation of 5.6%, and an average interassay coefficient of 8.2%. Method accuracy determined by spike recovery averaged 102.2%, and linearity determined by serial dilution averaged 106.9%. The serum–saliva correlation for DHEA in a combined male/female normative database (Salimetrics, LLC) is high ($r = 0.86$, $p < 0.0001$, $n = 39$). Mean ± SE DHEA concentrations were 229.7 ± 14.7 pg/mL.

Similarly, samples were assayed for salivary DHEAS in duplicate using a highly sensitive enzyme immunoassay. The test uses 100 µL of saliva per determination, has a lower limit of sensitivity of 43 pg/mL, standard curve range from 189 pg/mL to 15,300 pg/mL, an average intra-assay coefficient of variation of 7.3%, and an interassay coefficient of variation of 7.6%. Method accuracy determined by spike recovery averaged 105.9%, and linearity determined by serial dilution averaged 98.2%. Mean ± SE DHEAS concentrations were 4390.8 ± 403.9 pg/mL.

Testosterone

This assay was performed in duplicate using a highly sensitive enzyme immunoassay. The test uses 25 µL of saliva per determination, has a lower limit of sensitivity of 1.0 pg/mL, standard curve range from 6.1 pg/mL to 600 pg/mL, an average intra-assay coefficient of variation of 4.6%, and an average interassay coefficient of variation of 9.8%. Method accuracy determined by spike recovery averaged 104.3%

and linearity determined by serial dilution averaged 102.4%. Serum–saliva correlations from a normative database (Salimetrics, LLC) of male subjects is high ($r = 0.91$, $p < 0.001$, $n = 26$). Mean ± SE testosterone concentrations in this sample were 120.2 ± 5.6 pg/mL.

Nerve Growth Factor

This assay was performed in triplicate using a highly sensitive enzyme immunoassay. The standard curve measured NGF from 3.9 to 250 pg/mL. The assay has an intra-assay precision of 14.5% and an interassay precision of 15.5%. Recovery of NGF added to saliva samples averaged 95.3%. Linearity ranged from 82.3 to 127.2%. Mean ± SE NGF concentrations in this sample were 88.7 ± 13.3 pg/mL.

Alpha Amylase

Though mainly involved in starch digestion in the oral cavity,²⁶ sAA increases under physically and psychologically stressful conditions and is a correlate of sympathetic nervous activity.^{27,28} In this study, all samples were assayed via kinetic reaction. The assay employs a chromogenic substrate, 2-chloro-p-nitrophenol, linked to maltotriose. The enzymatic action of alpha amylase on this substrate yields 2-chloro-p-nitrophenol, which is spectrophotometrically measured at 405 nm using a standard laboratory plate reader. The amount of alpha amylase activity present in the sample is directly proportional to the increase (over a 2-minute period) in absorbance at 405 nm. Results are computed in units per milliliter of alpha amylase using the formula: [Absorbance difference per minute × total assay volume (328 mL) × dilution factor (200)]/[millimolar absorptivity of 2-chloro-p-nitrophenol (12.9) × sample volume (0.008 ml) × light path (0.97)]. Intra-assay variation computed for the mean of 30 replicate tests was less than 7.5%. Interassay variation computed for the mean of average duplicates for 16 separate runs was less than 6%. Mean ± SE sAA concentrations were 101.6 ± 15.5 U/mL.

Cortisol

All samples were assayed for salivary cortisol in duplicate using a highly sensitive enzyme immunoassay. The test uses 25 µL of saliva per determination, has a lower limit of sensitivity of 0.003 µg/dL, standard curve range from 0.012 µg/dL to 3.0 µg/dL, an average intra-assay coefficient of variation of 3.5%, and an average interassay coefficient of variation of 5.1%. Method accuracy determined by spike recovery averaged 100.8%, and linearity determined by serial dilution averaged 91.7%. Serum–saliva correlations from a normative database show the expected strong linear relationship, ($r = 0.91$, $p < 0.0001$, $n = 47$). Mean ± SE cortisol concentrations were 0.13 ± 0.9 µg/dL.

Data Analysis

Preliminary analysis incorporated the use of normal probability and residual plots to assess compliance with the assumptions

of linear regression. These plots revealed that NGF and sAA were positively skewed. These analytes were then log-transformed, which normalized each distribution.²⁹ Next, bivariate correlations explored relationships between independent variables (DHEA and DHEAS), dependent variables (testosterone, NGF, and sAA), and potential covariates (age, body mass index [BMI], years of military service, sampling time, and salivary cortisol concentrations). Finally, to quantify the unique and combined influence of DHEA and DHEAS on each endpoint, separate multiple linear regression analyses were conducted with DHEA and DHEAS as independent variables, and testosterone, NGF, and sAA as dependent variables, respectively. Where warranted, covariates were included in the model. Where applicable, hypothesis tests were based on log-transformed data; untransformed means are reported for ease of interpretation. All hypothesis tests were two-sided and the probability of committing a type I error was set at 0.05. It was acknowledged when more stringent conventional alpha levels were achieved ($p < 0.01$ or $p < 0.001$).

RESULTS

Subject Characteristics

Mean \pm SE age, BMI, and years of military service for this sample were 26.4 ± 0.7 years, 26.3 ± 0.6 kg/m 2 , and 5.7 ± 0.7 , respectively. Most subjects were Caucasian (78.6%). More than half had a high school education (57.1%), whereas the remainder possessed a 4-year or advanced degree. A broad cross section of military occupational specialties was represented. Combat experience was endorsed by 42.9% of subjects.

Selection of Covariates

Covariates are typically selected based on a theoretically supported influence upon the dependent variable of interest.³⁰ Age ($r = -0.33$, $p < 0.05$), education level ($\rho = -0.41$, $p < 0.01$), and years of military service (-0.43 $p < 0.01$) were associated with lower salivary testosterone concentrations. As expected, years of military service was highly correlated to (and presumably a function of) age ($r = 0.83$, $p < 0.001$). Thus, age and education were selected as covariates in the regression model examining testosterone as the dependent variable. Sampling time (which was restricted to a 1-hour time frame, detailed above) did not influence any of the end points (all $p > 0.05$), nor did salivary cortisol concentrations (all $p > 0.05$). Thus, neither was included as a covariate in the regression models.

Anabolic, Neuroprotective, and Neuroactive Effects of DHEA(S)

In the first regression model, the independent variables (DHEA and DHEAS) and covariates (age and education) combined to explain 23.4% of variance in testosterone ($F = 4.0$, $p < 0.01$). Inspection of the standardized beta coefficients revealed that DHEA exerted an independent effect on

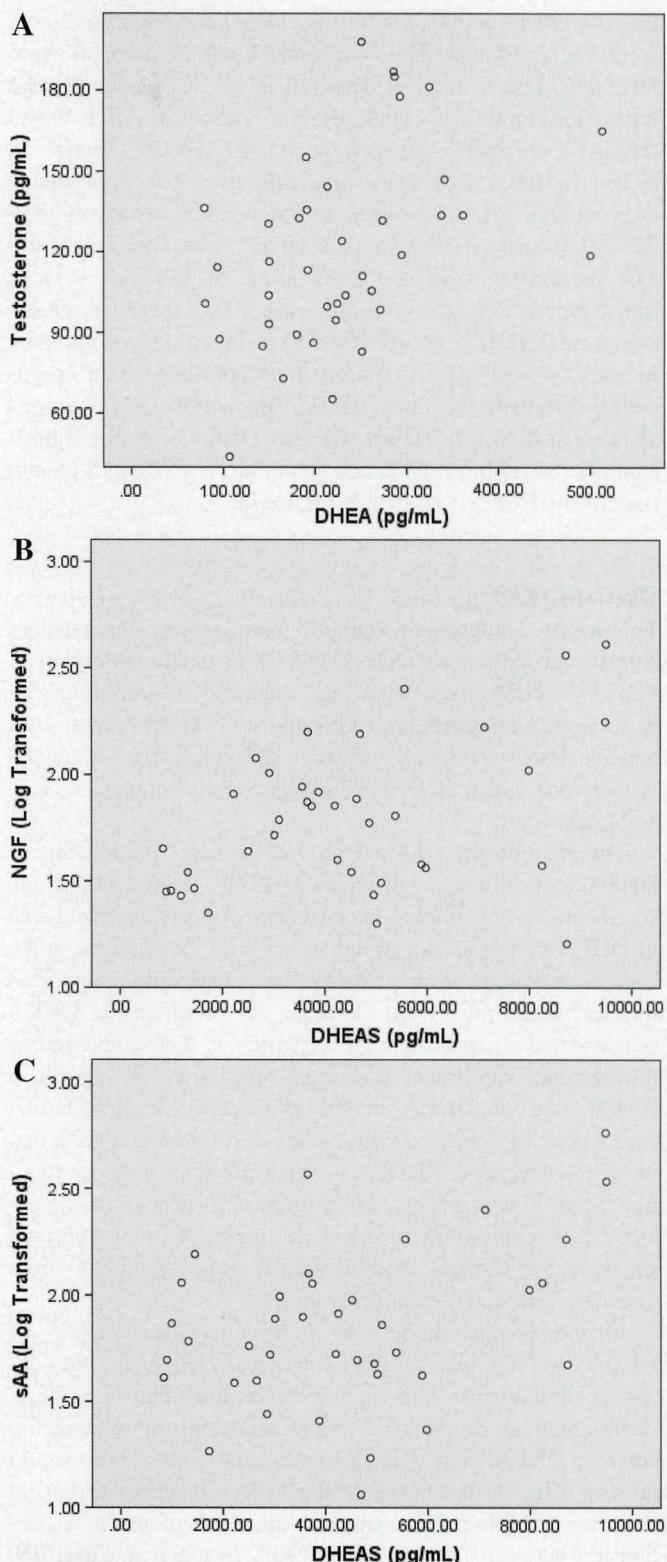


FIGURE 2. Bivariate associations between (A) DHEA and testosterone ($r = 0.45$, $p < 0.01$), (B) DHEAS and NGF ($r = 0.45$, $p < 0.01$), and (C) DHEAS and sAA ($r = 0.34$, $p < 0.05$).

testosterone ($\beta = 0.40, p < 0.01$), whereas the other predictors were not significant. The unadjusted bivariate association of DHEA to testosterone is depicted in Fig. 2. In the second regression model, the independent variables (DHEA and DHEAS) combined to account for 17.2% of variance in NGF ($p < 0.01$). Inspection of standardized beta coefficients showed that DHEAS exerted an independent effect on NGF ($\beta = 0.48, p < 0.01$), whereas DHEA was not significant. The unadjusted bivariate association of DHEAS to log-transformed NGF is shown in Figure 2B. In the third regression model, DHEA and DHEAS explained 7.4% of variance in sAA ($p = 0.09$). Inspection of standardized beta coefficients demonstrated that DHEAS independently influenced sAA ($\beta = 0.36, p < 0.05$), whereas DHEA was not significant. The unadjusted bivariate association of DHEAS to log-transformed sAA is shown in Figure 2C.

DISCUSSION

This study characterized anabolic, neuroprotective, and neuroactive properties of DHEA and DHEAS in military men. Salivary DHEA and DHEAS combined to substantially predict salivary testosterone, NGF, and, to a lesser extent, sAA concentrations. Moreover, DHEA independently influenced testosterone, whereas DHEAS independently influenced NGF and sAA.

The first model clarified an independent relationship of DHEA to testosterone, whereas DHEAS did not contribute significantly. This finding is consistent with the theorized role of DHEA as a precursor to the sex steroids, as reflected in the human steroidogenesis pathway and empirically supported throughout the preclinical literature. As noted earlier, DHEA is converted to androstenedione, which is then converted to testosterone. The observed strength of relationship suggests a central role of DHEA in the production of testosterone irrespective of age or educational level. It further suggests that its sulfated version—DHEAS—may not play a direct functional role in this process. More research is needed to characterize this relationship across sexes, under acute and chronic stress, and in clinical conditions such as posttraumatic stress disorder, TBI, and metabolic syndrome.

In the second model, an independent relationship of DHEAS to NGF was suggested, whereas DHEA did not contribute significantly. This finding differs from that of Schulte-Herbrüggen et al's human study showing no relationship between DHEAS and NGF in 40 pregnant women (who would possess different hormonal profiles). As alluded to earlier, at least two possible mechanisms are supportive of an active relationship between DHEA(S) and NGF. In the first, DHEA(S) binds with NGF TrkA and p75^{NTR} receptors to regulate apoptosis of target cells (e.g., sympathetic and sensory neurons).⁶ In the second, DHEA upregulates NGF mRNA expression in target cells (e.g., hippocampal cells).¹⁴ The first mechanism suggests shared biological "action" of DHEA(S) and NGF, whereas the second suggests that DHEA(S) may mediate the

"production" of NGF. Specifically, mRNA is transcribed from a DNA template within the nucleus and then carries coding information to the ribosomes for protein synthesis. Although the observed positive association of DHEAS and NGF is supportive of this latter mechanism, the cross-sectional design and noninvasive methodology of this study preclude definitive conclusions.

The third model suggested an independent influence of DHEAS on sAA, whereas DHEA did not contribute significantly. Produced in the oral cavity, sAA is described as a reliable correlate of circulating catecholamines, particularly norepinephrine, under both baseline and stress-induced conditions.^{27,28} Thus, it is advocated as a noninvasive and easily obtained salivary analogue of sympathetic tone.²⁵ The neuroexcitatory effects of DHEA(S) are believed to emanate partly from its role as a negative modulator of GABA (discussed earlier), which inhibits both norepinephrine¹⁷ and epinephrine.¹⁸ Inhibition of GABA-mediated neurotransmission, then, initiates a net excitatory effect.⁷ That DHEAS associated more substantially with sAA in this study complements some animal studies suggesting that DHEAS modulates the GABA_A receptor complex with greater potency than DHEA.^{19,20} Interestingly, negative modulators of the GABA_A receptor complex typically possess anxiogenic (i.e., anxiety-inducing) qualities,³¹ but this does not appear to be the case for DHEA(S). Several studies, in fact, document anxiolytic (i.e., anxiety-reducing) effects of DHEA(S) in chronically stressed individuals,^{32,33} which some authors attribute to its previously mentioned antiglucocorticoid properties.⁷

Limitations of this study should be addressed. First, this translational study involves measurement of peripheral hormone concentrations in humans. Concentration of a given hormone, however, lacks precise mechanistic information regarding its functional status (e.g., action on target receptors). Also, a single sample gives limited information in analytes known to possess a diurnal rhythm. Testosterone, cortisol, and DHEA typically peak shortly after waking and reach a nadir in the evening. By contrast, sAA typically reaches a nadir approximately 30 minutes after awakening and then steadily increases throughout the day.²⁶ DHEAS remains relatively stable across the day, whereas the diurnal pattern of NGF is not well understood. To mitigate this limitation, sampling occurred at a time point during which dramatic fluctuation is not expected, and was restricted to a single hour. Statistical analyses further showed that sampling time (within the single hour) did not associate with salivary concentrations of any of the analytes. Regardless, prospective studies evaluating these relationships across the diurnal cycle could provide more nuanced information.

In summary, this study examined unique and shared relationships of DHEA and DHEAS to biomarkers of anabolic, neuroprotective, and sympathetic nervous activity. It was demonstrated that DHEA and DHEAS possess anabolic, neuroprotective, and neuroexcitatory properties in military men, yet they appear to have distinct relationships with each end

point. Thus, separate study of DHEA and DHEAS activity is warranted. This work has broad implications for stress inoculation, TBI rehabilitation, and regenerative medicine in military personnel. For example, DHEA(S)' antiglucocorticoid properties may help military personnel not only to buffer acute stress reactions but also to manage the deleterious effects of chronic stress which may include immunosuppression, depression, fatigue, cognitive decline, and sleep disruption. Likewise, the observed link between DHEAS and NGF is particularly exciting with respect to potential advancements in TBI treatment as well as regenerative medicine for sensory and sympathetic systems. The association of DHEAS to sAA (the sympathetic analogue) may have less obvious clinical implications; coupled with its known covariance with cortisol, this may allude to a protective role within the complex, coordinated "fight or flight" response. Together, these relationships highlight the importance of not only characterizing endogenous DHEA(S) but also the potential impact of DHEA(S) supplementation in military personnel. In this author's view, well-designed randomized controlled trials documenting its efficacy and pinpointing its risks and side effects are needed to realize its preventive and therapeutic potential.

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14. ABSTRACT <p>Evidence links dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) to crucial military health issues, including operational stress, resilience, and traumatic brain injury. This study evaluated the anabolic, neuroprotective, and neuroexcitatory properties of DHEA(S) in healthy military men. A salivary sample was obtained from 42 men and assayed for DHEA(S), testosterone, nerve growth factor (NGF; which supports nerve cell proliferation), and salivary alpha-amylase (sAA; a proxy of sympathetic nervous system function). Separate regression analyses were conducted with DHEA and DHEAS as independent variables, and testosterone, NGF, and sAA as dependent variables, respectively. The models explained 23.4% of variance in testosterone ($p < 0.01$), 17.2% of variance in NGF ($p < 0.01$), and 7.4% of variance in sAA ($p = .09$). Standardized beta coefficients revealed that DHEA independently influenced testosterone ($\beta = 0.40$, $p < 0.01$), while DHEAS independently influenced NGF ($\beta = 0.48$, $p < 0.01$) and sAA ($\beta = 0.36$, $p < 0.05$). DHEA demonstrated anabolic properties, while DHEAS demonstrated neuroprotective and neuroexcitatory properties in military men. This area of study has broad implications for stress inoculation, traumatic brain injury rehabilitation, and regenerative medicine in military personnel.</p>				
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